IL-1β induction of MUC5AC gene expression is mediated by CREB and NF-κB and repressed by dexamethasone

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Mucin glycoproteins (mucins) are the major macromolecular components of lung mucus (45) and are responsible for its viscoelasticity (32, 46). Mucins are highly O-glycosylated proteins and are now identified by their protein backbone, which is encoded by one of 22 MUC genes. Twelve mucins—MUC1, 2, 3, 4, 5AC, 5B, 6, 7, 8, 13, 16, and 19—are expressed at the mRNA or protein level in lung epithelial cells or tissues (11, 47). However, MUC5AC and MUC5B, the secretory gel-forming mucins, are the most prominent mucins in lung mucus and airway secretions from nondiseased airways (27). They are overproduced in lung diseases: MUC5AC in asthma (39); MUC5B in status asthmaticus (51), chronic obstructive pulmonary disease (26), and idiopathic pulmonary fibrosis (50); and both MUC5AC and MUC5B in CF (27) and CF exacerbations (21). MUC5AC expression is typically restricted to goblet cells and MUC5B to glandular cells in the conducting epithelia of healthy airways (42). However, in chronic lung diseases, MUC5AC is also expressed in glandular cells (5) and MUC5B in goblet cells (7), suggesting that inflammatory mediators, in addition to upregulating mucin gene expression, can also alter their cellular expression, thereby further impacting mucin overproduction [reviewed in (47, 57)].

Inflammatory mediators commonly upregulated during respiratory inflammatory responses [interleukin-1β (IL-1β), IL-6/IL-17, tumor necrosis factor alpha (TNFα)] and epidermal growth factor receptor (EGFR) ligands (EGF, TGFα, neuregulin) increase MUC5AC mRNA abundance in human lung epithelial cells and transcriptionally upregulate MUC5AC gene expression [reviewed in (30)]. Studies from several laboratories have also shown that PKC→MEK→ERK→RSK is a predominant signal transduction pathway activated following ligand/receptor interactions in various lung epithelial cells and that EGFR is a predominant receptor [reviewed in (30, 47, 57)]. However, the EGFR-induced signaling that is primarily responsible for MUC5AC upregulation in the NCI-H292 lung cancer cell line is absent in primary human bronchial epithelial (HBE) cells (23), suggesting that other ligand receptor interactions may be important in primary HBE cells.

The cytokine IL-1β, one of the earliest mediators secreted during a proinflammatory response (13), is present at high levels in the lungs of patients with chronic lung diseases (52). Exposure of lung epithelial cells to IL-1β increases MUC5AC mRNA abundance in several respiratory tract cell lines, including the lung cancer NCI-H292 cell line (25, 54) and the HBE1 transformed cell line (16), as well as primary differentiated human nasal epithelial (HNE) cells (54), and...
HBE cells (16, 19, 20). The IL-1β-induced upregulation of the MUC5AC gene has been reported to be mediated by cAMP response element-binding protein (CREB) or nuclear factor-κB (NF-κB) transcription factor binding to cognate cis sites in the MUC5AC promoter with CREB binding to a cAMP response element (CRE) cis site (−878 nt) in H292 cells (54) and NF-κB subunits binding to a NF-κB cis site (−3594 nt) in the distal promoter in the HBE1 cell line (16). However, upregulation of MUC5AC expression by IL-1β-activated transcription factors in primary differentiated HBE cells, which differentiate to mimic a conducting airway epithelium (18, 59), has not been reported, and mucin gene regulation in primary differentiated HBE cells can differ from that in cell lines (23). Thus we utilized primary differentiated HBE cells and the A549 lung adenocarcinoma cell line to further investigate the contribution of NF-κB and CREB to the IL-1β-induced upregulation of MUC5AC gene expression.

We also investigated the response of MUC5AC in these cells when exposed to both IL-1β (upregulated in diseased airways) and to glucocorticoids (clinically used in the treatment of chronic lung diseases as an anti-inflammatory therapeutic strategy). Studies on mucin gene expression in cells exposed to inflammatory mediators and glucocorticoids are limited. Previously, we have shown that the glucocorticoid dexamethasone (Dex) utilizes two glucocorticoid responsive elements (GRE) to transcriptionally repress MUC5AC gene expression both in A549 cells (8) and in primary differentiated HBE cells (6) under homeostatic conditions. Because the impact of glucocorticoids on mucin gene expression in cells exposed to inflammatory mediators may have clinical relevance but has not been systematically studied, we also investigated whether Dex represses MUC5AC gene expression in lung epithelial cells exposed to IL-1β.

MATERIALS AND METHODS

Cell culture. A549 cells [American Type Culture Collection (ATCC), Manassas, VA] were grown as previously described (8). A549 stocks were analyzed for short tandem repeats (STR) using the Promega Powerplex 16 system and an ABI 3130XL with GenemapperID software. The profiles were identical to each other and matched the profile for A549 in the ATCC STR database.

Normal primary HBE cells (lots 7F3421, 4F1289J, and 235243), HBE cell growth medium, and DMEM were purchased from Lonza (Walkersville, MD). HBE cells were amplified once on plastic, then plated on semipermeable Transwell membrane inserts (Fisher, Pittsburgh, PA) coated with human type IV collagen (Sigma, St. Louis, MO). Cells were grown submerged for 5–7 days until they reached 75–80% confluency and then under air-liquid conditions for 17 days, as previously described (6).

Exposure to mediators. Differentiated HBE or A549 cells were exposed to IL-1β (R&D Systems, Minneapolis, MN), IL-1 receptor antagonist (IL-1Ra) (ImgeneX, San Diego, CA), or Dex (Sigma) at concentrations and times indicated in the figure legends. Mediators (resuspended in PBS) were added to both compartments of differentiated HBE cells. HBE cells were maintained in hydrocortisone-free medium for 3 days prior to exposure to Dex. A549 cells were maintained in serum-free media 24 h prior to the addition of mediators. Cells exposed to PBS alone were used as controls.

Gene expression. RNA isolation and analysis was performed as previously described (6). MUC5AC and actin mRNA levels were quantified by real-time PCR (qPCR) using SYBR Green (Bio-Rad Laboratories, Hercules, CA) and normalized to β-actin mRNA levels. Fold changes were determined by comparing levels of mediator-exposed cells to control levels.

Chromatin immunoprecipitation (ChIP) and re-ChIP analyses. ChIP analyses were carried out as previously reported (6) according to the manufacturer’s protocol (Upstate, Charlotteville, VA) except that nuclear lysates were used. DNA-protein complexes were cross-linked, immunoprecipitated with the first antibody and then immunoprecipitated with the second antibody (43). DNA was isolated from the immunocomplexes and PCR or qPCR was carried out. Values were normalized by input DNA. Results were expressed as the mean fold change over basal levels.

Statistical analysis. Data were evaluated for significance using the Student’s t-test for straightforward comparisons or the one-way ANOVA test using the Bonferroni method (Prism software; GraphPad, San Diego, CA) for multiple comparisons within an experiment. Differences were considered significant at P < 0.05.

RESULTS

IL-1β increases MUC5AC mRNA abundance in lung epithelial cells via the IL-1β receptor. Primary differentiated HBE and A549 cells were exposed to increasing concentrations of IL-1β (1, 10, and 100 ng/ml) for 24 h. A significant increase (P < 0.05) in MUC5AC mRNA abundance was observed at all concentrations with differentiated HBE cells; the increase plateaued at 10 ng/ml (Fig. 1A). The increase in MUC5AC mRNA abundance in A549 cells was significant at 10 and 100 ng/ml (Fig. 1B). A time-course assay showed that MUC5AC mRNA increased slightly but significantly at 4 h (P < 0.05) in HBE cells, and was markedly increased (P < 0.001) at 6 h in both cell types (Fig. 1, C and D). Although MUC5AC levels remained elevated in HBE cells following IL-1β exposure at 24 h (Fig. 1C), there was a significant decrease in expression levels in A549 cells at the 24-h time point (Fig. 1D). These data demonstrated that two different types of lung epithelial cells exhibit increased MUC5AC mRNA levels following exposure

<table>
<thead>
<tr>
<th>MUC5AC primer region</th>
<th>Reference</th>
<th>Forward primer 5’→3’</th>
<th>Reverse primer 5’→3’</th>
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</thead>
<tbody>
<tr>
<td>Proximal, TATA; nt: −254 to +50</td>
<td>(31)</td>
<td>caggggaggtctcagggagt</td>
<td>cattgtgtagcgccggcggggag</td>
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<tr>
<td>Mucin regulatory domain, nt: −1022 to −759</td>
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<tr>
<td>Distal: nt: 3685 to −3540</td>
<td>(16)</td>
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<td>ccgctgacccctgagag</td>
</tr>
<tr>
<td>3’-Untranslated region</td>
<td>(4)</td>
<td>gcacgcagagggagt</td>
<td>ccacactcccacacgaag</td>
</tr>
</tbody>
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Table 1. MUC5AC primers used for PCR and qPCR analyses
to IL-1β and that the IL-1β increase observed earlier was sustained in HBE cells.

Both differentiated HBE cells and A549 cells have an IL-1 type I receptor (IL-1R) that responds similarly to IL-1β (10). To determine whether the IL-1β-induced increase in MUC5AC abundance was mediated through IL-1R, A549 cells were preexposed to increasing concentrations of an IL-1R antagonist (IL-1Ra) for 10 min, followed by a 6-h exposure to 10 ng/ml of IL-1β (Fig. 2). At the highest concentration used (250 ng/ml), IL-1Ra alone did not alter MUC5AC mRNA abundance, demonstrating the lack of endogenous IL-1β in the proprietary growth media and showing that IL-1R is not necessary for MUC5AC baseline expression. Complete blockade of IL-1R was achieved with 250 ng/ml IL-1Ra, a concentration that resulted in a significant decrease in the IL-1β-induced increase in MUC5AC expression, which was reduced to the level observed during exposure of cells to IL-1Ra alone.

IL-1β signaling increases binding of RNA polymerase to the TATA box domain in the MUC5AC promoter. To demonstrate that IL-1β transcriptionally upregulates MUC5AC expression, we utilized ChIP assays to evaluate the binding of RNA Pol II to specific domains in the MUC5AC promoter (Fig. 3A) following exposure to IL-1β. Temporal analyses indicated that Pol II binding was increased at the MUC5AC proximal promoter region that contained the TATA box and the transcriptional start site (31) following exposure to IL-1β at 1 h, and remained significantly elevated (P < 0.05) for the next 17 h (Fig. 3B). In contrast, Pol II binding at a region upstream of the TATA box [defined as a mucin regulatory domain (MRD) in the MUC5AC promoter] was observed, but levels did not change following IL-1β exposure (Fig. 3C). Furthermore, in a control experiment, Pol II binding was not detectable in the 3′-untranslated region (UTR) domain of the MUC5AC gene (4) at baseline or following IL-1β exposure (data not shown). Because histone acetylation often accompanies increased Pol II activity, the levels of acetylated H4 in the TATA box region of the MUC5AC promoter were also evaluated. ChIP analysis showed increased acetylated H4 levels following exposure of cells to IL-1β for 18 h (Fig. 3D). These data indicated that IL-1β induces an increased presence of Pol II and modification of chromatin structure at the MUC5AC proximal promoter containing the TATA box and transcriptional start site, which
CREB assays (EMSAs) have demonstrated binding of CREB to a mucin regulatory domain (MRD) in the MUC5AC promoter. Electrophoretic mobility shift assays (EMSA) demonstrated whether or not binding of transcription factors occurs at specific cis sequences in situ in target gene promoters (35). We had previously used ChIP analysis to show that Dex-activated glucocorticoid receptor (GR) binds to the promoter (6), which is upstream of MUC5AC promoter, as shown in (Fig. 9). Both sites are within the region covered by primers used for GRE3 ChIP analysis (Fig. 3). Additionally, we had previously identified two potential NF-kB sites (GGGACCTTTC and GGGACTGCTC) at −975 nt and −957 nt in the MUC5AC promoter upstream of the GRE3 and CRE sites. EMSA data demonstrate that the −975 nt site is implicated in binding of p65, the subunit that contains the transcription activation domain of NF-kB, when A549 cells are exposed to TNFα (37). Both TNFα and IL-1β increase MUC5AC expression in H292 cells (54), and CREB and NF-kB are both implicated in the IL-1β-induced upregulation of MUC5AC in H292 cells (55). Thus we exposed A549 and primary differentiated HBE cells to IL-1β and used ChIP analyses to investigate binding of CREB and of p65 to the domain that contains both the CRE (∼871 nt) and NF-kB (∼973 nt) sites.

ChIP analyses of A549 cells exposed to IL-1β for 1, 2, 4, or 18 h demonstrated that CREB and p65 bind minimally to that domain under basal conditions and that binding of both CREB and p65 were increased following 2 h of exposure to IL-1β (Fig. 4A). Re-ChIP assays were subsequently performed to determine whether both transcription factors were simultaneously recruited to the same domain. Data demonstrated that CREB and p65 were simultaneously recruited to the same domain following 2 h of IL-1β exposure in A549 cells (Fig. 4B, lane 5). The CREB signal following IL-1β exposure increased (Fig. 4B, lanes 1 and 3), however, saturation of the PCR bands in the absence of IL-1β was reached, precluding semiquantitation. Therefore, to avoid saturation effects in experiments in which cell numbers were limited, we utilized qPCR in experiments with HBE cells (Fig. 5).

Primary differentiated HBE cells were exposed to IL-1β for 2 h, and ChIP analyses were performed. Data demonstrated binding of CREB and of NF-kB (Fig. 5A). A ChIP and re-ChIP experiment demonstrated that CREB and p65 were simultaneously recruited to the same domain at 2 h following IL-1β exposure (Fig. 5B). This domain in the MUC5AC promoter, which has a functional CRE and a new functional NF-kB cis site activated by IL-1β, is termed an MRD (see Fig. 9).

IL-1β-mediated NF-kB p50 and p65 subunit binding in the MUC5AC promoter is temporally regulated. ChIP analysis showed p65 binding to an NF-kB site in the MRD of the MUC5AC promoter following IL-1β exposure to A549 and differentiated HBE cells (Figs. 4 and 5). A recent study shows that HBE1 cells exposed to IL-1β results in the presence of p65 and p50 in the flanking regions around a distal NF-kB site.

Contribute to the observed IL-1β-mediated increase in MUC5AC transcription.

IL-1β induces simultaneous binding of CREB and p65 to an MRD in the MUC5AC promoter. Electrophoretic mobility shift assays (EMSA) have demonstrated binding of CREB to a CRE cis site (∼878 nt) in the MUC5AC promoter in the NCI-H292 lung cancer line following IL-1β exposure (54). Although EMSAs show that transcription factors in nuclear extracts can bind to synthetic oligonucleotides of defined cognate sequences in promoter targets in a test tube, ChIP assays demonstrate whether or not binding of transcription factors occurs at specific cis sequences in situ in target gene promoters (35). We had previously used ChIP analysis to show that Dex-activated glucocorticoid receptor (GR) binds to the GRE3 site in the MUC5AC promoter (6), which is upstream of the CRE site (Fig. 9). Both sites are within the region covered by primers used for GRE3 ChIP analysis (Fig. 3). Additionally, we had previously identified two potential NF-kB sites (GGGACCTTTC and GGGACTGCTC) at −975 nt and −957 nt in the MUC5AC promoter upstream of the GRE3 and CRE sites. EMSA data demonstrate that the −975 nt site is implicated in binding of p65, the subunit that contains the transcription activation domain of NF-kB, when A549 cells are exposed to TNFα (37). Both TNFα and IL-1β increase MUC5AC expression in H292 cells (54), and CREB and NF-kB are both implicated in the IL-1β-induced upregulation of MUC5AC in H292 cells (55). Thus we exposed A549 and primary differentiated HBE cells to IL-1β and used ChIP analyses to investigate binding of CREB and of p65 to the domain that contains both the CRE (∼871 nt) and NF-kB (∼973 nt) sites.

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Differentiated HBE cells were exposed to IL-1β and whether both NF-κB subunits bind to each site, we investigated the response of cells when exposed to both mediators. Dexamethasone represses constitutive and IL-1β-mediated induction of MUC5AC expression. The glucocorticoid Dex reduces MUC5AC mRNA abundance in NCI-H292 (22), A549 (8, 33), and differentiated HBE (6) cells under basal conditions. However, it is not known whether Dex likewise reduces MUC5AC mRNA levels in cells exposed to IL-1β. Because both Dex and IL-1β transcriptionally mediate MUC5AC, we investigated the response of cells when exposed to both mediators. Differentiated HBE cells were exposed to IL-1β (10 ng/ml) or vehicle for 18 h followed by increasing concentra-
To further elucidate the effects of IL-1β and Dex on MUC5AC gene expression, A549 cells were preexposed to IL-1Ra or vehicle, followed by a 6-h exposure to IL-1β and/or Dex (100 nM) (Fig. 8). Comparisons between IL-1β + Dex, IL-1β + IL-1α, and IL-1β + IL-1α + Dex showed that MUC5AC mRNA levels were statistically decreased \( (P < 0.001) \). IL-1β-mediated MUC5AC upregulation, which was previously demonstrated to be reduced by IL-1α blockade (250 ng/ml IL-1Ra; Fig. 2), was further attenuated \( (P < 0.05) \) by subsequent exposure to Dex. Thus the combination of IL-1α blockage and Dex additively inhibited the ability of IL-1β to upregulate MUC5AC expression, suggesting distinct mechanisms of repression of MUC5AC gene expression by Dex and induction by IL-1β.
MUC5AC mucin, initially called tracheobronchial mucin (34), is a major component of lung mucus and is copiously produced during acute and chronic lung diseases (27), thereby greatly contributing to disease morbidity and mortality. MUC5AC overproduction is in part a response to the presence of inflammatory mediators, and studies have been carried out using various lung cell types (cancer, transformed, primary) with most occurring in NCI-H292 cells [reviewed in (47)]. The prevailing concept is that most inflammatory mediators increase MUC5AC expression in primary differentiated HBE cells. Furthermore, we show that functionally blocking IL-1R prevents the IL-1β-induced upregulation of MUC5AC gene expression in A549 cells, and predictably, in differentiated HBE cells. Both these cell types express IL-1R type I, but not IL-1R type II, and have similar responses to IL-1β (10).

The IL-1β-induced regulation of the MUC5AC gene is mediated at the transcriptional level; initial studies in NCI-H292 cells show that IL-1β exposure to these cells resulted in binding of the transcription factor CREB to a CRE cis site (~878 nt) in the MUC5AC promoter (54). Exposure of differentiated HBE cells to prostaglandin F2α (9) and ATP (53) likewise activates CREB binding to this CRE cis site, demonstrating its importance in the upregulation of MUC5AC. Our studies confirm that IL-1β activates binding of CRE to this CRE site.

Importantly, we have shown that IL-1β also activates binding of NF-κB to a cognate cis site upstream of the CRE site in the MRD (Fig. 9). We had previously shown this NF-κB site was activated following TNFα exposure to A549 cells (37). Our ChiP and re-ChiP experiments demonstrated that IL-1β simultaneously recruits both NF-κB and CREB to the MRD, thus identifying the MRD as a novel and important site in the MUC5AC promoter for IL-1β upregulation of MUC5AC in both HBE and A549 cells. Interactions of NF-κB and CREB in the IL-1β-induced upregulation of MUC5AC in H292 cells have recently been suggested by immunoprecipitation and Western blot studies, although ChiP analyses were not performed (55).

Analysis of the MUC5AC promoter indicates the presence of several additional potential NF-κB sites (Fig. 9). IL-1β, which activates NF-κB (36), activates two of these sites in the MUC5AC promoter: −975 nt (this study) and −3594 nt (16). The latter study shows that NF-κB p50 and p65 subunits are detectable in the MUC5AC distal promoter region following a 20-min exposure of IL-1β to the HBE1 A549 cancer cell line and confirm IL-1β-induced upregulation in primary differentiated HBE cells. Furthermore, we show that functionally blocking IL-1R prevents the IL-1β-induced upregulation of MUC5AC gene expression in A549 cells, and predictably, in differentiated HBE cells. Both these cell types express IL-1R type I, but not IL-1R type II, and have similar responses to IL-1β (10).

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Fig. 9. Schema of the MUC5AC promoter. Rectangles show putative cAMP response element (CRE) (hatched) and NF-κB (dark gray) cis sites. Triangles above the promoter indicate functional CRE (hatched triangle) (54), NF-κB (dark gray triangle) (16), and glucocorticoid responsive elements (GRE) (white triangle) (6, 8) cis sites. The expanded domain containing two known functional (CRE, GRE3) sites and a newly identified functional NF-κB site is termed a mucin-regulatory domain (MRD). TSS, transcriptional start site.

cell line. The study also demonstrates p50 binding to the distal NF-κB site at 1 h following IL-1β exposure; however, binding of p65, which contains the transactivation domain of NF-κB, was not assessed. Although we had demonstrated binding of p65 in the MRD in A549 cells (Fig. 4A) and HBE cells (Fig. 5A) at 2 h, we had not assessed p50 binding. Thus we carried out ChIP assays in both A549 and HBE cells and methodically evaluated direct binding of the p50 and p65 subunits to the NF-κB sites in the MUC5AC MRD and the distal promoter at 1 and 2 h (Fig. 6). These data demonstrate that IL-1β-activated signaling results in NF-κB binding to both the distal NF-κB site and to the newly identified NF-κB site in the MUC5AC MRD in A549 cells and in primary differentiated HBE cells. Specifically, these data show that p50 binding predominates in both cell types at 1 h post IL-1β exposure, as previously demonstrated in HBE cells (16). Furthermore, the present investigation demonstrates that continued exposure (2 h) to IL-1β differentially alters the occupancy of both the distal and MRD NF-κB sites in A549 cells compared with HBE cells, resulting in recruitment of p65, which contains the transactivation domain of NF-κB, to both NF-κB sites in A549 and HBE cells. This suggests that the switch to the p50/p65 heterodimer in HBE cells and p65 homodimer in A549 cells with continued IL-1β exposure is what subsequently results in IL-1β-mediated transcriptional activation of the MUC5AC gene. The inability of IL-1β to increase MUC5AC expression at the 1-h time point in either A549 or HBE cells (Fig. 1, C and D) may be explained by the current paradigm that p50 homodimers have the potential to downregulate NF-κB targeted gene regulation, whereas p50/p65 heterodimers activate transcription (1, 49). The differences in NF-κB binding observed between HBE and A549 cells are not necessarily surprising because p50 and p65 have specific functions in individual genes and cell types (17). That p65 homodimers predominate at two NF-κB sites in A549 cells and p50/p65 heterodimers in HBE cells is a significant observation that supports the importance of primary cell models to confirm transcriptional regulation of genes initially studied in lung cancer cell lines. In summary, this is the first report to demonstrate that a specific inflammatory mediator, IL-1β, activates different NF-κB regulatory domains in the MUC5AC promoter and that there is a temporal dependence of activation of NF-κB subunits in multiple domains. Because the CRE site in the proximal promoter is also temporally activated, the potential for multiple cis sites to be activated by two transcription factors in a time-dependent fashion increases the complexity of transcriptional regulation of the MUC5AC gene.

The interactions that occur in lung epithelial cells simultaneously exposed to inflammatory mediators (upregulate mucin gene expression) and anti-inflammatory agents such as glucocorticoids (repress mucin gene expression) are clinically relevant but challenging to unravel mechanistically. Indeed, the impact of Dex alone on MUC5AC gene expression in quiescent NCI-H292 cells is controversial because Dex reduces MUC5AC mRNA levels (22), but also has now been reported...
to not alter MUC5AC mRNA or protein levels (56). However, Dex and the classical steroids budesonide and fluticasone reduce MUC5AC mRNA levels induced by TGFα (EGFR ligand) or a combination of TGFα and poly I:C in NCI-H292 cells (56). Our data show that Dex and IL-1β differentially affect MUC5AC mRNA levels, consistent with antagonizing effects on MUC5AC when lung epithelial cells are exposed to a combination of Dex and IL-1β. Dex (100 and 1,000 nM) represses the IL-1β-activated upregulation of MUC5AC gene expression in primary differentiated HBE cells, the concentrations of which approximate those in the lungs of patients treated with glucocorticoids (14).

The mechanisms by which Dex decreases MUC5AC gene expression when cells are exposed to IL-1β have not been established, but predictably involve interactions at the MRD in the MUC5AC promoter (Fig. 9) of CREB and NF-κB, transcription factors activated by IL-1β, as well as GR. Dex-activated GR binds to the GRE3 site in the MRD in A549 (8) and primary differentiated HBE cells (6) to repress MUC5AC gene expression. GRE3 is a negative (n) GRE cis site because of its homology with the 3′ end of the GRE consensus sequence palindrome (3). The MRD can be classified as a composite GRE domain (i.e., an nGRE site adjacent to cis sites known to bind inflammatory transcription factors) (12, 15, 38). Because IL-1β induces simultaneous binding of CREB and NF-κB to cis sites that flank the GRE3 site in the MRD domain, and because Dex represses MUC5AC expression in cells exposed to IL-1β, there may be competition between CREB and/or NF-κB and GR at the MUC5AC promoter that inhibits binding of inflammatory transcription factors to ultimately favor GR binding and thus gene repression. Clearly, additional studies are required to investigate whether such interactions occur at the MUC5AC promoter.

Alternatively, interactions between GR and NF-κB can occur (40), and such interactions may prevent inflammatory transcription factors from undergoing nuclear translocation and/or binding to cis sites in the MUC5AC promoter. Dex appears to further block MUC5AC expression in conditions of no possible IL-1β activation (i.e., with full IL-1R blockade), suggesting a separate mechanism of additional Dex-mediated repression beyond simply interfering with proinflammatory stimulation at the MUC5AC promoter. Future experiments with dissociative steroids [e.g., steroids with which GRE binding and anti-inflammatory properties can be separated (48)] may be informative because GR activated by dissociative ligands predictably will not affect GR binding to GRE sites, but instead interact directly with inflammatory transcription factors in the nucleus or cytoplasm (41). Undoubtedly, further elucidation of the molecular mechanisms responsible for mucin gene control mediated by cytokines prevalent in diseased airways and by steroids used for treatment will be fundamental for refining and reinventing current therapeutic strategies in these common airway disorders of inflammatory mucin overproduction.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

12. Delosscher K, Vanden Bergh W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator pro-


